Diffusion in Embryogenesis

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A simple order-of-magnitude calculation suggests that diffusion may be the underlying mechanism in establishing morphogenetic gradients in embryonic development.

It has been a great surprise and of considerable importance to find that most embryonic fields seem to involve distances of less than 100 cells, and often less than 50.
Professor Lewis Wolpert[1]

WHEN I read this sentence I was delighted because it seemed to confirm some conclusions I had come to on purely theoretical grounds[2]. It is an old idea that "gradients" are involved in embryological development —in fact, C. M. Child[3] in 1941 wrote a whole book on the subject. Many of the gradients to which Child referred seem more likely, in retrospect, to be the results of development rather than its cause. An outsider to embryology has the impression that in recent years gradients have become a dirty word. This is partly because of the failure to isolate unambiguously the molecules involved, whose concentration is presumed to constitute the gradient, and partly because a feeling has grown up that diffusion is not a fast enough mechanism for establishing gradients. In this article I aim to show that this fear is unfounded, and, on the contrary, that the known facts, sparse as they are, fit rather well to a mechanism based on diffusion. The problem can be stated in this way: what is the maximum distance over which a steady concentration gradient could plausibly be set up in the times available during the development of the embryo?

The obvious model for setting up a simple gradient is illustrated in Fig. 1. At one end of a line of cells one postulates a source—a cell which produces the chemical (which I shall call a morphogen) and maintains it at a constant level. At the other end the extreme cell acts as a sink: that is, it destroys the molecule, holding the concentration at that point to a fixed low level. The morphogen can diffuse from one cell to another along the line of cells. After a time the system approaches a dynamic equilibrium, and it is easy to show that if the effective diffusion constant is everywhere the same, the concentration gradient will be linear.

Of course, real embryological structures will have three dimensions, but if for convenience we restrict ourselves to sheets of cells such as, for example, the insect epidermis[4] or the developing amphibian retina[5] the problem becomes two-dimensional. The source can be considered to be a line of cells (the line being perpendicular to the paper in Fig. 1) and similarly for the sink, thus reducing the problem to one dimension.

It is not difficult to calculate how long it would take to set up such a system, supposing that both the source and the sink are turned on at time zero. Diffusion is a random walk process, and the dimensions of the diffusion constant, D, are L^2T^{-1} (where L is length and T is time). This should be contrasted with a mechanism having a velocity (with dimensions LT^{-1}) as proposed, for example, by Goodwin and Cohen[6]. Because in diffusion the length enters as the square, pure diffusion processes are very rapid over rather short distances (say, the size of a cell) and very slow over long distances (say, the size of an organ).

The concentration approaches its final value asymptotically, so one must have some criterion for deciding whether the gradient at any time is sufficiently close to a straight line. I have arbitrarily taken the gradient to be effectively established when it is everywhere within ΔC of the final value, and chosen ΔC as 1 per cent of C_0 (C_0 is the maximum value at the origin). It would make little difference to the argument if ΔC were considered to have half this value.

The gradient might be set up in various ways. The result in each case can be expressed as

$$t = \frac{A\ (nl)^2}{D}$$

where t = time in seconds to set up the gradient; n = number of cells between source and sink; l = length of each cell, in cm; and D = diffusion constant, in $cm^2\ s^{-1}$. A is a numerical constant, the exact value of which will depend on the way the gradient is developed.

Mathematically the simplest way is to start with zero concentration of the morphogen everywhere at time zero, and thereafter to maintain the source at concentration C_0 and the sink at concentration zero. This gives a value of A of 0·42. Biochemically more realistic models give values only a little larger than this, so a good general value for A would be 0·5. It was pointed out to me by Dr Aaron Klug that, if the initial concentration were uniformly $C_0/2$, the time required is reduced to a little less than one-quarter, and A will have a value of about 0·09. More realistic models of this general sort give values of A of, say, 0·15. The calculations of A were carried out by Mrs Mary Munro.

In what follows, I shall assume that A is 0·5 (the simple mechanism), but it should be remembered that the organism might be able to reduce this to about a third of this value.

The diffusion constant in water for all but the smallest molecules—provided they are roughly spherical—is inversely proportional to their mean radius, to a near approximation. Thus, increasing the molecular weight by a factor of 1,000, from, say, a small organic molecule such as ATP (mol. wt. 507) to a very large protein like polynucleotide polymerase (mol. wt. about $0{\cdot}5 \times 10^6$) reduces the diffusion constant only by a factor of 10. Now it is reasonable to expect that the morphogen will diffuse rather rapidly, and should be able to pass fairly efficiently from cell to cell. It is also likely to be a rather specific molecule. For these reasons I doubt if morphogens

will turn out to be large proteins or common ions[7] like K^+ or Na^+. An obvious choice would be an organic molecule of about the size of, say, cyclic AMP or a steroid. That is, with a molecular weight in the range 300 to 500. The diffusion constant[8] in water (at 20° C) for such a molecule is about 4 or 5×10^{-6} cm^2 s^{-1}. (The diffusion of salts like NaCl or KCl is about three or four times as fast as this.)

The inside of a cell is very far from being made of water, and one must estimate the effective diffusion constant within a cell. This amounts to estimating the effective viscosity. The cytoplasm being a concentrated mixture of molecules having a large variety of sizes, the relative viscosity will be considerably higher than water at the same temperature. For a small molecule, which can, as it were, slip between many of the other molecules, the effective viscosity is unlikely to be as big as the bulk viscosity of the cytoplasm (wherever that may be). It is difficult to make any precise estimate of the effective diffusion constant, which in any case may vary considerably between different types of cell. Allowing a factor of increase of viscosity of $\times 6$ (corresponding to a sucrose solution 40 per cent by weight), which seems not unreasonable[9], would make the effective diffusion constant about $0{\cdot}8 \times 10^{-6}$ cm^2 s^{-1}.

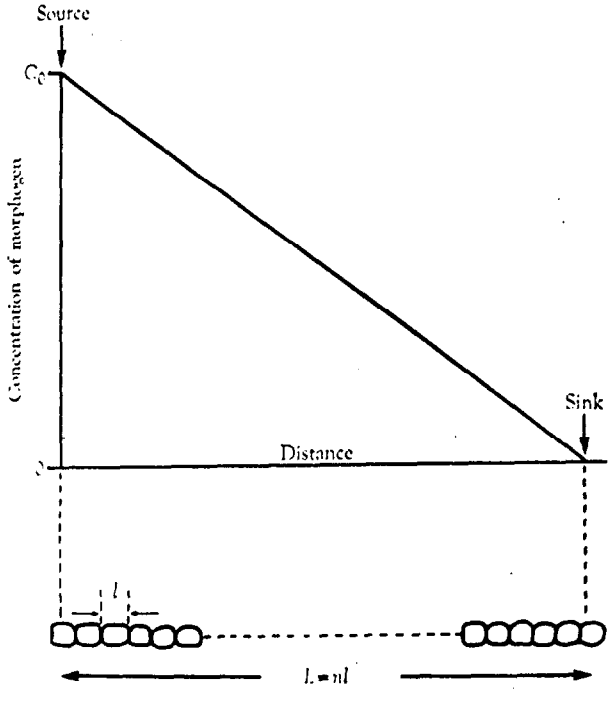

Fig. 1. To illustrate how a source and a sink can produce a linear gradient of concentration. Each cell in the line has length l cm. The distance between source and sink is L cm. Because n is the number of cells in the line, $L = nl$.

How does the morphogen get from cell to cell? It would seem inefficient to make all cell membranes easily permeable to it. To do this it would in any case have to be very small and rather hydrophobic[10]. Moreover, a high general permeability would allow the molecule to escape too easily from the tissue and this would not only disturb the gradient but possibly interfere in other parts of the organism. One is therefore driven to postulate a special mechanism which allows a relatively quick passage of the morphogen from one cell to another in the tissue of interest. Whether this involves tight junctions or other special structures remains to be seen.

Such a mechanism could be facilitated diffusion. This is characterized by being independent of energy, specific (usually stereospecific) and saturatable at high concentrations of the diffusing molecule. The passage of glucose through the membrane of a human red blood cell or an ascites tumour cell is believed to have this character.

At low concentrations (that is, far from saturation) the mechanism can be described by a permeability, P, measured in cm s^{-1}. It is easy to show that the effective diffusion constant, D', for our problem is given by $1/D' = 1/D + 1/Pl$, where l is the length of each cell in the direction of diffusion[11]. Thus, if D is $0{\cdot}8 \times 10^{-6}$ cm^2 s^{-1} and $l = 10$ μm (say) we see that if the "resistance" to flow of the morphogen because of permeability between cells was equal to that due to diffusion within a cell, P would have to have the value 8×10^{-4} cm/second. This is a high value, but probably not impossibly high[12]. If we arbitrarily take P as about half this and D as before, we obtain $D' \simeq 0{\cdot}27 \times 10^{-6}$ cm s^{-1}.

We now need an experimental estimate of the time needed to set up a gradient. This is not easy to obtain. Most embryologists would feel that a day is too long. A minute seems far too short. A few hours would seem about right—Wolpert has suggested that between 5 to 10 h is not unreasonable for many of the well-studied cases (ref. 1, page 41). I shall assume a figure of 10^4 s (approximately 3 h), because some time must be allowed for the changes which take place after the gradient is set up.

Combining our formulae we obtain

$$t = An^2l^2\left(\frac{1}{D} + \frac{1}{Pl}\right)$$

and substituting the chosen values: $t = 10^4$ s, $A = 0{\cdot}5$, $l = 10$ μm, $D = 0{\cdot}8 \times 10^{-6}$ cm^2 s^{-1}, $P = 4 \times 10^{-4}$ cm s^{-1} we obtain $n \simeq 70$ cells. If l were 30 μm, n would come to a little over thirty cells. Even allowing for the very approximate nature of the calculations, the agreement with the figures given in the quotation at the start of the article is striking. In broad outline what the calculation shows is that, for the times considered, distances or the order of a millimetre (or less) are possible, but distances of a centimetre are too great[13]. Of course, for organisms which develop very rapidly the distances would have to be smaller than a millimetre.

We can take it, then, that assuming the effective viscosity of cytoplasm has not been grossly underestimated, and provided there is a special mechanism to increase the rate of permeability of the morphogen between cells, there are many cases in embryology where the times and distances involved are quite compatible with a mechanism based on diffusion. It is important, however, to make two reservations. There may be special cases, involving setting up gradients quickly over large distances (of the order of several centimetres) which may require other mechanisms, such as the signalling devices suggested by Goodwin and Cohen[9]. Cases of "mushroom growth" (as, for example, the growth of mushroom) are unlikely to be due to diffusion alone. Secondly, in one's enthusiasm for diffusion, it is important to realize that the many other problems remain to be tackled. Even when the gradient has been set up the cell has to recognize it. Because at least in the insect integument the "gradient" appears to impose a polarity on those epidermal cells which become scales and bristles[14] the cells must have some additional mechanism (involving microtubules?) to do this.

In the case of the amphibian eye the retinal ganglion cells must not only recognize the presumed gradient (so that they know where they are in the retina); each cell must also convey this information to the far end of its growing axon, so that it can make the connexion at the appropriate place on the optic tectum[5]. Moreover, there are likely to be subsidiary mechanisms to guide the growing bundle of nerves along the right path to the tectum.

In two brilliant articles published about ten years ago, Locke[15] showed that the pattern of wrinkles obtained on

the adult cuticle of Rhodnius after operations on the earlier larval stages (usually the last larval stage) can only be explained by a "gradient" of some sort, running from one intersegmental membrane to the next, and repeating in successive segments. He showed convincingly that neither mechanical expansion alone, nor polarity alone, could explain the results. Dr Locke kindly sent us the original photographs of some of his material. Mrs Mary Munro and I, together with Dr Peter Lawrence, have attempted to fit these patterns to a pure diffusion model. Although, following Locke's arguments, the observed wrinkles have roughly the expected pattern, they differ in detail from the computed pattern. Moreover, various estimates of the diffusion constant disagree drastically. We are therefore currently exploring a model in which each cell in the epidermis attempts to maintain the concentration of the morphogen within itself to a previously preset level, determined soon after the gradient is first set up. This model, which has only one disposable parameter, is a much better fit with Locke's data. More elaborate hypotheses, within the basic diffusion framework, are also under consideration. It is important, therefore, not to approach these problems with too naïve a model[16].

Finally, one should emphasize that gradients are unlikely to command general acceptance until their biochemical basis is discovered experimentally, and that this may not prove an easy task. Mathematically minded biologists could well object that any theory which has the same mathematical formalism would necessarily fit the observed patterns, and that the agreement between the calculated and observed distances (on the diffusion theory sketched above) may only be coincidental. In spite of these possible objections it is my belief that mechanisms based on diffusion are not only plausible but rather probable. Nature usually has such difficulty evolving elaborate biochemical mechanisms (for example, those used in protein synthesis) that the underlying processes are often rather simple. If this approach serves to make the idea of diffusion gradients respectable to embryologists it will have served its purpose.

I thank my wife for drawing the figure, my colleagues, especially Dr Peter Lawrence and Mrs Mary Munro, for many helpful discussions, and Professors Lewis Wolpert and W. D. Stein for sending me information.

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[1] Wolpert, L., *J. Theoret. Biol.*, **25**, 1 (1969). This article, entitled "Positional Information and the Spatial Pattern of Cellular Differentiation", should be consulted both for a modern statement of the problem and also for references to earlier work.

[2] The basic idea of this article was presented at a lecture given to students at the Fourth NATO Advanced Study Institute of Molecular Biology in July 1969 at Spetsai.

[3] Child, C. M., *Patterns and Problems of Development* (Chicago University Press, Chicago, 1941).

[4] For a review see Lawrence, P. A., *Adv. Insect Physiol.* (in the press). See especially Stumpf, H., *Roux Arch. Ent. Mech. Org.*, **158**, 315 (1967).

[5] For a review see Gaze, R. M., *Growth and Differentiation*, *Ann. Rev. Physiol.*, **29**, 59 (1967).

[6] Goodwin, B., and Cohen, M. H., *J. Theoret. Biol.*, **25**, 49 (1969).

[7] The mechanism for forming a source and a sink for ions also presents special problems, whereas for organic molecules rather simple enzymatic processes could do the trick.

[8] The effect of temperature on the viscosity of water does not seriously affect the calculations. Taking the viscosity of water (in arbitrary units) as 1·0 at 20° C, its value at 5° C is about 1½ and at 39° C is close to 2/3.

[9] See, for example, a very ingenious fluorescent method used by Victor W. Burns (*Biochem. Biophys. Res. Commun.*, **37**, 1008; 1969) using a small organic molecule. He obtained a factor of about ×6 for Euglena. The figure for yeast was about twice this.

[10] Lieb, W. R., and Stein, W. D., *Nature*, **224**, 240 (1969).

[11] This assumes that the permeability is fairly evenly distributed over the cell membrane. If it were concentrated in a small patch the effective diffusion would be slower.

[12] For example, P for glucose in ascites cells at 37° C in about $4{\cdot}5 \times 10^{-4}$ cm/s (Kolber, A. R., and LeFevre, P. G., *J. Gen. Physiol.*, **50**, 1907; 1967), or in human red cells at 37° C about 1×10^{-4} cm/s (Millar, D. M., *Biophys. J.*, **5**, 407; 1965). Admittedly these are among the higher values of P known so far. See Stein, W. D., *The Movement of Molecules Across Cell Membranes*, chap. 4 (Academic Press, New York, 1967). It should be remembered that in going from one cell to the next the morphogen may have to cross two cell membranes.

[13] An upper limit can be calculated assuming that the morphogen is an ion, that it diffuses (at 37° C) in the cytoplasm as freely as in water, that the permeability between cells is so high that it does not slow down the process at all, and that the most efficient method is used to set up the gradient. The distance then comes to 1·5 cm, but I feel that this combination of assumptions is quite unrealistic.

[14] Piepho, H., *Naturwissenschaften*, **42**, 22 (1955); Lawrence, P. A., *J. Exp. Biol.*, **44**, 607 (1966).

[15] Locke, M., *J. Exp. Biol.*, **36**, 459 (1959); *J. Exp. Biol.*, **37**, 398 (1960).

[16] The present model could be elaborated by assuming two different morphogens, one having a gradient sloping from left to right, and the other with a gradient from right to left: on this model the position of a cell would be characterized by the ratio of its concentration of the two morphogens. This particular elaboration, however, would not significantly alter any of the arguments given in this article.